The mitigating effect of calcification-dependent of utilization of inorganic carbon of Chara vulgaris Linn on NH$_4$-N toxicity

Heyun Wang, Leyi Ni $^*$, Ping Xie $^*$

Donghu Experimental Station of Lake Ecosystems, State Key Laboratory for Freshwater Ecology and Biotechnology of China, Institute of Hydrobiology, The Chinese Academy of Sciences, Donghu South Road 7, Wuhan 430072, People's Republic of China

**Highlights**
- The effect of external HCO$_3^-$ and NH$_4$-N stress on calcareous plants was assessed.
- External HCO$_3^-$ promoted calcification and decreased the increase of solution pH.
- Calcification alleviated the toxicity of NH$_4$-N by regulating solution pH.
- Calcification was dependent of utilization of dissolve inorganic carbon.
- The mitigating effect of HCO$_3^-$ on NH$_4$-N toxicity is dependent of plant calcification.

**ABSTRACT**
Increased ammonium (NH$_4$-N) concentrations in water bodies have been reported to adversely affect the dominant species of submerged vegetation in meso-eutrophic waters worldwide. However calcareous plants were lowly sensitive to NH$_4$-N toxicity.

In order to make clear the function of calcification in the tolerance of calcareous plants to NH$_4$-N stress, we studied the effects of increased HCO$_3^-$ and additional NH$_4$-N on calcification and utilization of dissolve inorganic carbon (DIC) in Chara vulgaris Linn in a 7-d sub-acute experiment (light:dark 12:12 h) carried out in an open experimental system in lab. Results revealed that calcification was dependent of utilization of dissolve inorganic carbon. Additional HCO$_3^-$ significantly decreased the increase of pH while additional NH$_4$-N did not. And additional HCO$_3^-$ significantly improved calcification while NH$_4$-N did in versus in relation to the variation of DIC concentration. However, addition of both HCO$_3^-$ and NH$_4$-N increased utilization of DIC. This resulted in calcification to utilization of DIC ratio decreased under additional NH$_4$-N condition while increased under additional HCO$_3^-$ conditions in response to the variation of solution pH.

In the present study, external HCO$_3^-$ decreased the increase of solution pH by increasing calcification, which correspondingly mitigated the toxic effect of high NH$_4$-N. And we argue that the mitigating effect of increased HCO$_3^-$ on NH$_4$-N toxicity is dependent of plant calcification, and it is a positive feedback mechanism, potentially leading to the dominance of calcareous plants in meso-eutrophic water bodies.

*Corresponding authors. Tel./fax: +86 27 68780622.
E-mail addresses: nily@ihb.ac.cn (L. Ni), xieping@ihb.ac.cn (P. Xie).

© 2013 Elsevier Ltd. All rights reserved.
Dissolve inorganic carbon (DIC) were one of the most important environmental variables that affected the morphology and distribution of Chara plants (McConnaughey, 1998b), due to its respectively fast attenuation and limited solubility in water column. Although intra-specific and inter-specific variations of external encrustations on the cell wall are present within the genus Chara (Anadón et al., 2002), obvious calcification was found to be on the stem of thalli inhabiting water relatively high alkalinity (Kufel and Kufel, 2002). According to trans-calcification, Ca\textsuperscript{2+} – 2H\textsuperscript{+} exchange catalyzed by Ca\textsuperscript{2+} ATPase is involved in calcification of Chara (McConnaughey and Falk, 1991). This indicates the function of calcification as a proton generator. Since 1990s, lots of studies had demonstrated the benefits for macrophytes from calcification as a proton generator related to bicarbonate utilization (e.g. McConnaughey, 1991; McConnaughey and Falk, 1991), phosphorus assimilation (e.g. McConnaughey and Whelan, 1997; Siong, 2006; Siong and Asaeda, 2009b) and hyper-accumulator of heavy metals (McConnaughey, 1991; Siong and Asaeda, 2009a). In mildly alkaline water, DIC decreased by two means, either was taken up by the photosynthesis of the algae or became incorporated in the cell wall of Chara by calcification (Ray et al., 2003). However, lots of experiments studying bicarbonate utilization of Chara spp. usually do not quantify calcification or even mention its existence (McConnaughey, 1998). Therefore, experimental studies to establish quantitative relationship between calcification and the utilization of DIC are demanded to clarify its mechanism. In previous studies, rates of calcification and photosynthesis of Chara cell were estimated from changes in total alkalinity (TA) and dissolved inorganic carbon (DIC) of solution in an enclosing experimental system. Obviously, both TA and DIC were significantly correlated with solution pH, which result in the dependence of calcification to photosynthesis ratio on solution pH in the closed experimental system (McConnaughey, 1991, 1998; McConnaughey and Whelan, 1997). In the present study, a sub-acute experiment was conducted in a open experimental system to examine the combined effects of addition of both NH\textsubscript{4}+ and HCO\textsubscript{3}– on calcification to photosynthesis ratio for C. vulgaris by testing variation of pH in solution, utilization of DIC and calcification of plants. To this aim we tested the following hypotheses: (1) additional NH\textsubscript{4}+ would decreased calcification by decreased solution pH; (2) additional HCO\textsubscript{3}– would increase calcification although decreased the increase of solution pH and (3) calcification to utilization of DIC ratio decreased under additional NH\textsubscript{4}+ condition while increased under increased HCO\textsubscript{3}– conditions. The function of calcification in the tolerance of calcareous plants to NH\textsubscript{4}– stress was discussed in relation to solution pH.

2. Method and materials

2.1. Plant culture

Chara vulgaris Linn, a polymorphic charophyte, has a broad, world-wide distribution between 70°N and 50°S and is prone to accumulate lime and form apparently non-banded encrustation on the stems (Anadón et al., 2002), with more than 60% of ash mass containing lime and form apparently non-banded encrustation on the stems (Anadón et al., 2002). According to trans-calcification, Ca\textsuperscript{2+} – 2H\textsuperscript{+} exchange catalyzed by Ca\textsuperscript{2+} ATPase is involved in calcification of Chara (McConnaughey and Falk, 1991). This indicates the function of calcification as a proton generator. Since 1990s, lots of studies had demonstrated the benefits for macrophytes from calcification as a proton generator related to bicarbonate utilization (e.g. McConnaughey, 1991; McConnaughey and Falk, 1991), phosphorus assimilation (e.g. McConnaughey and Whelan, 1997; Siong, 2006; Siong and Asaeda, 2009b) and hyper-accumulator of heavy metals (McConnaughey, 1991; Siong and Asaeda, 2009a). In mildly alkaline water, DIC decreased by two means, either was taken up by the photosynthesis of the algae or became incorporated in the cell wall of Chara by calcification (Ray et al., 2003). However, lots of experiments studying bicarbonate utilization of Chara spp. usually do not quantify calcification or even mention its existence (McConnaughey, 1998). Therefore, experimental studies to establish quantitative relationship between calcification and the utilization of DIC are demanded to clarify its mechanism. In previous studies, rates of calcification and photosynthesis of Chara cell were estimated from changes in total alkalinity (TA) and dissolved inorganic carbon (DIC) of solution in an enclosing experimental system. Obviously, both TA and DIC were significantly correlated with solution pH, which result in the dependence of calcification to photosynthesis ratio on solution pH in the closed experimental system (McConnaughey, 1991, 1998; McConnaughey and Whelan, 1997). In the present study, a sub-acute experiment was conducted in a open experimental system to examine the combined effects of addition of both NH\textsubscript{4}+ and HCO\textsubscript{3}– on calcification to photosynthesis ratio for C. vulgaris by testing variation of pH in solution, utilization of DIC and calcification of plants. To this aim we tested the following hypotheses: (1) additional NH\textsubscript{4}+ would decreased calcification by decreased solution pH; (2) additional HCO\textsubscript{3}– would increase calcification although decreased the increase of solution pH and (3) calcification to utilization of DIC ratio decreased under additional NH\textsubscript{4}+ condition while increased under increased HCO\textsubscript{3}– conditions. The function of calcification in the tolerance of calcareous plants to NH\textsubscript{4}– stress was discussed in relation to solution pH.

2.2. Experimental design

It was a factorial design of 2 × 2 of dissolve inorganic carbon (DIC) and ammonia nitrogen (NH\textsubscript{4}–N). NH\textsubscript{4}–N included 2 levels: low level (without additional NH\textsubscript{4}–N) and high level (additional 1.0 mg L\textsuperscript{-1} NH\textsubscript{4}–N as NH\textsubscript{4}Cl); and HCO\textsubscript{3}– level also included two degree: low level (without additional HCO\textsubscript{3}–) and high level (additional 0.5 mM HCO\textsubscript{3}– as NaHCO\textsubscript{3}). Therefore, four treatments were recorded as CK (no addition), +N (additional 1.0 mg L\textsuperscript{-1} NH\textsubscript{4}–N), +C (additional 0.5 mM HCO\textsubscript{3}–) and +N+C (additional 1.0 mg L\textsuperscript{-1} NH\textsubscript{4}–N and 0.5 mM HCO\textsubscript{3}–). Under every treatment conditions, four flasks with plants were used to assess the difference of treatments and two flasks without plants were used to evaluate the changes of solution with time. In consequence, twenty-four 250 mL conical flasks were placed in the plant incubator with an illumination at the water surface of 120 μ mol m\textsuperscript{-2} s\textsuperscript{-1} and with a 12:12 photoperiod under temperature condition of 25 ± 2 °C. Then every 4–5 apical tips was weighed and transferred to sixteen flasks with 200 mL culture solution when light photoperiod began. The pH of these solutions was adjusted to 8.2 by 0.1 M HCl or 0.1 M NaOH before moved to these flasks. And the solution were replenished each day at the start of the light period. The plants were clearly rinsed with ultrapure water before transplanted to new solution. Seven days later, all the plants were harvested.

2.3. Experimental sampling and measurement

In order to study the daily variation of calcification rate, the sampling was carried out at both 4 h and 8 h of the first photoperiod. For the other 6 d, 20 mL solution were fetch and frozen in –20 °C only at 8 h of the photoperiod, 10 mL for dissolve inorganic carbon (DIC) and 10 mL for ion content measurement. The pH of these solutions was measured daily just after the sampling of 8 h. The dissolve inorganic carbon (DIC) content of solution was measured with TOC analyzer (O.I. analytical 1010, USA). Analyses of cation content were performed with a Dionex DX100 ion chromatograph equipped with a 25-μl sample loop, a cation-exchange column and a suppressed conductivity detection system. For separation, an ion Pac CG10A guard column (50 × 4 mm) was coupled to an IonPac CS10A analytical column (250 × 4 mm). For suppressor, a Dionex CRS ultra self-regeneration suppressor was employed and 2.0 software was used for system control and data acquisition. All IC related equipment was supplied by Dionex (DX-100, USA).

2.4. Calculation and analysis

2.4.1. Calculation

Both dissolved inorganic carbon (DIC) uptake and Ca\textsuperscript{2+} uptake by plants in each sample was calculated as the difference of DIC (or Ca\textsuperscript{2+}) content in the solution with plants and the means of DIC
(or Ca\(^{2+}\)) content in two flasks without plants. In order to avoid the effect of fresh weight of plants before experiment, the utilization rate of DIC at the first 4th hour and 8th hour were expressed as µmol C h\(^{-1}\) g of fresh weight (DIC\(_{\text{uptake}}\)) and calcification rate was defined as µmol Ca h\(^{-1}\) g of fresh weight (Ca\(_{\text{uptake}}\)). Total utilization of DIC and calcification were calculated as the sum of utilization of DIC and calcification every days, expressed as µmol C g\(^{-1}\) and µmol Ca g\(^{-1}\), respectively.

2.4.2. Analysis

The concentration of DIC in solution was analyzed using a three-way ANCOVA with day, NH\(_4\)-N and HCO\(_3\) as fixed factors and with fresh weight of plants as covariance. DIC\(_{\text{uptake}}\) and Ca\(_{\text{uptake}}\) and increased pH for the first day were analyzed using a three-way ANOVA with hour, NH\(_4\)-N and HCO\(_3\) as fixed factors. DIC\(_{\text{uptake}}\) and Ca\(_{\text{uptake}}\), and increased pH for the later 6 d were analyzed using a three-way ANOVA with day, NH\(_4\)-N and HCO\(_3\) as fixed factors. The correlation between total utilization of DIC and calcification was calculated by linear regression. The ratio of calcification to the utilization of DIC was analyzed using a two-way ANOVA with +C and hour under different NH\(_4\)-N and Ca\(^{2+}\) (\(+C\)) significantly increased DIC\(_{\text{uptake}}\) and calcification to the utilization of DIC was analyzed using a two-way ANOVA with NH\(_4\)-N and HCO\(_3\) as fixed factors. All data was analyzed by SPSS 13.0.

3. Results

3.1. Impact of treatments on concentration of DIC in solution

With fresh weight of plants as covariance, three-way analysis of ANCOVA showed that additional HCO\(_3\) (\(+C\)) significantly increased concentration of DIC in solution (\(F = 358.40, p < 0.001\)) and significant interaction with time (day) occurred in the whole experiment (\(F = 2.39, p < 0.05\)). However, additional NH\(_4\)-N (\(+N\)) did not significantly affect concentration of DIC in solution (\(F = 2.02, p > 0.05\)). Concentration of DIC at 8 h of each photoperiod decreased with day for all treatments (Fig. 1).

3.2. The variation of solution pH

Plants photosynthesis strongly increased pH of the contain solution for all treatments and solution pH with plants varied from 8 h to 10(Fig. 2). For the first day, additional HCO\(_3\) (\(+C\)) significantly reduced the increase of solution pH in contrast to CK treatment while additional NH\(_4\)-N (\(+N\)) did not (Fig. 2, Table 1). For the later 6 d, both time (d) and \(+N\) significantly promoted while \(+C\) reduced the increase of solution pH (Fig. 2, Table 2).

3.3. The utilization rate of dissolve inorganic carbon and calcification rate

Both \(+N\) treatment and \(+C\) treatment significantly improved the utilization rate of DIC (DIC\(_{\text{uptake}}\)) but no interaction occurred between them for the first day (Fig. 3a and b, Table 1). Significant interaction on DIC\(_{\text{uptake}}\) existed between hour and \(+C\), and between hour, \(+C\) and \(+N\) (Table 1), indicating different responses of DIC\(_{\text{uptake}}\) to the interaction of \(+C\) and hour under different NH\(_4\)-N treatments. For the later 6 d, three-way ANOVA analysis showed that the single effects on DIC\(_{\text{uptake}}\) of day, \(+N\) and \(+C\) were significant and interaction only significantly occurred between day and \(+C\) (Table 2). Ca\(_{\text{uptake}}\) differed between hours, indicating the daily variation of calcification rate. Both \(+N\) and \(+C\) significantly affected Ca\(_{\text{uptake}}\), interactive effect of \(+N\) and \(+C\) on Ca\(_{\text{uptake}}\) also occurred for the first day (Table 1). For the later 6 d, Ca\(_{\text{uptake}}\) was only significantly affected by day, \(+C\) and the interaction of day, \(+N\) and \(+C\) (Table 2).

3.4. Total utilization of dissolve inorganic carbon and calcification

Both total utilization of DIC and calcification of plants increased with time (day) (Fig. 4). Linear regression showed that calcification of plants were linearly related to total utilization of DIC by plants for all treatments (Fig. 5). Two-way ANOVA analysis showed that \(+C\) significantly increased the ratio of calcification to DIC utilization (\(F = 15.44, p < 0.01\)) while \(+N\) did not (\(F = 1.91, p > 0.05\)). No significant interaction on the ratio existed between \(+C\) and \(+N\) (\(F = 0.003, p > 0.05\)).

4. Discussion

4.1. The main factors that affect calcification of Chara plants

For Chara plants, calcification accompanies the photosynthetic utilization of HCO\(_3\) and calcification compete for DIC with photosynthesis. According to McConnaughey (1998), calcification rate of Chara thaili is mainly affected by light intensity, Ca\(^{2+}\) and DIC.
Table 1
Results of the three-way ANOVA models for the effects of hours (h), additional NH₄-N (N), additional HCO₃⁻ (C) and their interaction on the utilization rate of dissolve inorganic carbon (DIC uptake, μmol Ca h⁻¹ g⁻¹) and calcification rate (Ca²⁺ uptake, μmol Ca h⁻¹ g⁻¹) and the results of the two-way ANOVA models for the effects of additional NH₄-N (N), additional HCO₃⁻ (C) and their interaction on increased pH for the first day. Significant effects (p < 0.05) are indicated in bold.

<table>
<thead>
<tr>
<th></th>
<th>DIC uptake (μmol Ca h⁻¹ g⁻¹)</th>
<th>Ca²⁺ uptake (μmol Ca h⁻¹ g⁻¹)</th>
<th>pH</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>d.f.</td>
<td>F</td>
<td>P</td>
</tr>
<tr>
<td>h</td>
<td>1</td>
<td>2.649</td>
<td>0.117</td>
</tr>
<tr>
<td>N</td>
<td>1</td>
<td>18.976</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>h × N</td>
<td>1</td>
<td>0.124</td>
<td>0.727</td>
</tr>
<tr>
<td>C</td>
<td>1</td>
<td>79.147</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>C × C</td>
<td>1</td>
<td>10.19</td>
<td>0.004</td>
</tr>
<tr>
<td>Error (model)</td>
<td>24</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Table 2
Results of the three-way ANOVA models for the effects of day (d.), additional NH₄-N (N.), additional HCO₃⁻ (C.) and their interaction on the utilization rate of dissolve inorganic carbon (DIC uptake, μmol C h⁻¹ g⁻¹) and calcification (Ca²⁺ uptake, μmol Ca h⁻¹ g⁻¹) and increased pH for the later 6 d-dark photoperiods. Significant effects (p < 0.05) are indicated in bold.

<table>
<thead>
<tr>
<th></th>
<th>DIC uptake (μmol C h⁻¹ g⁻¹)</th>
<th>Ca²⁺ uptake (μmol Ca h⁻¹ g⁻¹)</th>
<th>pH</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>d.f.</td>
<td>F</td>
<td>P</td>
</tr>
<tr>
<td>d</td>
<td>5</td>
<td>12.223</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>N</td>
<td>1</td>
<td>8.602</td>
<td>0.005</td>
</tr>
<tr>
<td>d × N</td>
<td>5</td>
<td>1.079</td>
<td>0.379</td>
</tr>
<tr>
<td>C</td>
<td>1</td>
<td>59.398</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>d × C</td>
<td>5</td>
<td>5.358</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>N × C</td>
<td>1</td>
<td>1.021</td>
<td>0.316</td>
</tr>
<tr>
<td>d × N × C</td>
<td>5</td>
<td>2.286</td>
<td>0.055</td>
</tr>
<tr>
<td>Error (model)</td>
<td>72</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Fig. 3. Utilization rate of DIC (μmol C h⁻¹ g⁻¹) at 4 h (a) and 8 h (b), and calcification rate (Ca²⁺ uptake, μmol Ca h⁻¹ g⁻¹) at 4 h (c) and 8 h (d) for the first day in four different treatments: CK (no addition), +N (additional 1.0 mg L⁻¹ NH₄-N), +C (additional 0.5 mM HCO₃⁻) and +N+C (additional 1.0 mg L⁻¹ NH₄-N and 0.5 mM HCO₃⁻). Means ± SE (n = 4).
on solution pH. For significantly increased concentration. Our experimental results – alkalinity suggested stronger buffer capability (e.g. Kahara within +C treatment significantly increased calcification rate might result from increased calcification in relation to DIC concentration (McConnaughey, 1991). Lots of studies on coral had proved that calcification was independent of the concentration of DIC in solution (e.g. Furla et al., 2000). However, the independence of calcification on the content of DIC in solution was due to the sufficient inorganic carbon inside plants. In the present study, inorganic carbon inside the plants was exhaust during pre-treatment. Therefore the concentration of DIC inside plants for the first light–dark photoperiod was determined by the content of DIC in solution. Thus, increased HCO$_3^-$ within +C treatment significantly increased calcification rate for the first day (Fig. 3c, Table 1) by promoting the concentration of inorganic carbon inside plants (Fig. 1). Furthermore, this was why we departly analyzed utilization of DIC and calcification of the first day from that of the later 6 d.

Results from pH-drift experiments often showed that higher HCO$_3^-$ – alkalinity suggested stronger buffer capability (e.g. Kahara and Vermaat, 2003; Pagano and Titus, 2004; Pierini and Thomazb, 2004). This indicated addition of HCO$_3^-$ usually mitigated the increase of solution pH. In the present study, significant reduction of the increase of pH of the solution within +C treatment confirmed the mitigating effect of increased HCO$_3^-$ on solution pH. For calcareous Chara plants, buffer capability of HCO$_3^-$ is ascribed to H$^+$ secretion by calcification (e.g. McConnaughey and Falk, 1991; McConnaughey and Whelan, 1997; McConnaughey, 1998). Therefore the reduction of the increase of solution pH in response to addition of HCO$_3^-$ may result from increased calcification in relation to increased HCO$_3^-$ concentration. Our experimental results that increased calcification rate for the first day and increased total calcification within +C treatment (Figs. 3 and 4 Tables 1 and 2) conformed to the hypothesis 2. Similar result was also reported in coccolithophorid as that extant HCO$_3^-$ significantly increased calcification rate of Emiliania huxleyi Lohmann (Nimer and Merrett, 1992).

4.3. Calcification-depence of on the utilization of DIC in relation to solution pH

For calcareous plants, the level of interaction between photosynthesis and calcification was taxon dependent. It was reported that high competition for DIC between photosynthesis and calcification exists in Chara thalli whereas no competition was in Stylophora pistillata microcolonies (e.g. McConnaughey and Falk, 1991; Furla et al., 2000). In the present study, the utilization of DIC resulted from photosynthesis and calcification of plants. We speculate that the high dependence of calcification on the utilization of DIC (Fig. 5) in Chara thalli was greatly related to the ratio of calcification to photosynthesis (C/P). Furthermore, we speculate increased C/P would result in the increase of the ratio of calcification to the utilization of DIC. In details, +N increased utilization of DIC by photosynthesis, and meanwhile decreased DIC utilization by calcification (Figs. 3 and 4, Table 2). Therefore, addition of NH$_4^-$-N did not significantly decrease the increase of pH (Fig. 2). This indicated that the promoted effect of NH$_4^-$-N as nutrient source on plant photosynthesis was almost equal to its mitigating effect as the buffer of solution alkalinity. For calcareous Chara thalli, plant calcification competed for DIC with photosynthesis in mild–alkaline water (e.g. McConnaughey and Falk, 1991; Ray et al., 2003). So the increase of DIC utilization by photosynthesis would result in the decrease of available DIC for calcification. As discussed above, calcification was only affected by DIC concentration in the present study. Thus, we speculated decreased calcification rate within +N treatment for the first day (Fig. 3c and d) resulted from restriction of the DIC concentration. Significant decrease of calcification rate with hours suggested similar reason (Fig. 3c and d, Table 2). These results seemed to be contrary to previous studies that found the source of inorganic carbon used in calcification of Chara came from the plant body (e.g. McConnaughey, 1991). Lot of studies on coral had proved that calcification was independent of the concentration of DIC in solution (e.g. Furla et al., 2000). However, the independence of calcification on the content of DIC in solution was due to the sufficient inorganic carbon inside plants. In the present study, inorganic carbon inside the plants was exhaust during pre-treatment. Therefore the concentration of DIC inside plants for the first light–dark photoperiod was determined by the content of DIC in solution. Thus, increased HCO$_3^-$ within +C treatment significantly increased calcification rate for the first day (Fig. 3c, Table 1) by promoting the concentration of inorganic carbon inside plants (Fig. 1). Furthermore, this was why we departly analyzed utilization of DIC and calcification of the first day from that of the later 6 d.

Results from pH-drift experiments often showed that higher HCO$_3^-$ – alkalinity suggested stronger buffer capability (e.g. Kahara and Vermaat, 2003; Pagano and Titus, 2004; Pierini and Thomazb, 2004). This indicated addition of HCO$_3^-$ usually mitigated the increase of solution pH. In the present study, significant reduction of the increase of pH of the solution within +C treatment confirmed the mitigating effect of increased HCO$_3^-$ on solution pH. For calcareous Chara plants, buffer capability of HCO$_3^-$ is ascribed to H$^+$ secretion by calcification (e.g. McConnaughey and Falk, 1991; McConnaughey and Whelan, 1997; McConnaughey, 1998). Therefore the reduction of the increase of solution pH in response to addition of HCO$_3^-$ may result from increased calcification in relation to increased HCO$_3^-$ concentration. Our experimental results that increased calcification rate for the first day and increased total calcification within +C treatment (Figs. 3 and 4 Tables 1 and 2) conformed to the hypothesis 2. Similar result was also reported in coccolithophorid as that extant HCO$_3^-$ significantly increased calcification rate of Emiliania huxleyi Lohmann (Nimer and Merrett, 1992).

4.3. Calcification-depence of on the utilization of DIC in relation to solution pH

For calcareous plants, the level of interaction between photosynthesis and calcification was taxon dependent. It was reported that high competition for DIC between photosynthesis and calcification exists in Chara thalli whereas no competition was in Stylophora pistillata microcolonies (e.g. McConnaughey and Falk, 1991; Furla et al., 2000). In the present study, the utilization of DIC resulted from photosynthesis and calcification of plants. We speculate that the high dependence of calcification on the utilization of DIC (Fig. 5) in Chara thalli was greatly related to the ratio of calcification to photosynthesis (C/P). Furthermore, we speculate increased C/P would result in the increase of the ratio of calcification to the utilization of DIC. In details, +N increased utilization of DIC by photosynthesis, and meanwhile decreased DIC utilization by calcification (Figs. 3 and 4, Table 2). Therefore,
the ratio of calcification to the utilization of DIC decreased within +N treatment in contrast to that CK treatment (Fig. 5a and c). With HCO$_3^-$ addition, both photosynthesis and calcification increased, and resulted in increased utilization of DIC (Figs. 3a and b, and 4). However, extant carbon added as HCO$_3^-$ was preferentially incorporated into calcite (Sikes et al., 1980; McConnaughey and Gillikin, 2008). This would resulted in increased C/P in relation to addition of HCO$_3^-$, correspondingly resulted in the increase of calcification to DIC utilization ratio, as showed as Fig. 5a and c. It was obvious that our experimental results conformed to hypothesis 3.

In previous studies, calcification to photosynthesis ratio varied from 1 to 2 as a function of solution pH between 9 and 10 (McConnaughey et al., 1994; McConnaughey and Whelan, 1997; McConnaughey, 1998). This suggested the ratio of calcification to the utilization of DIC would varied between 0.5 and 0.67. In the present study, solution pH varied from 9 to 10. However, higher calcification to the utilization of DIC above 0.67 occurred in two HCO$_3^-$ addition treatments (Fig. 5c and d). A possible reason was that the utilization of DIC was underestimated because of the exchange of CO$_2$ between solution and atmosphere.

4.4. The mitigating effect of calcification on NH$_4^+$-N toxicity

Macrophyte responded to NH$_4^+$ typically by free amino acid accumulation (Van Katwijk et al., 1997; Cao et al., 2007, 2009a,b, 2011; Nimptsch and Pflugmacher, 2007; Van der Heide et al., 2008; Zhang et al., 2010, 2011), especially by Glutamine (Van der Heide et al., 2008), oxidative stress (Zhang et al., 2010, 2011), inhibited growth and structural tissue damage (Brun et al., 2002; Nimptsch and Pflugmacher, 2007). Among of these responses, the important significance of solution pH was emphasized by Van der Heide et al. (2008) for solution pH regulated NH$_3$ – NH$_4^+$ ratio, which correspondingly affected carbon–nitrogen balances (Van der Heide et al., 2008; Christianen et al., 2011). This indicated the decrease of solution pH from calcification would alleviated the toxic effect of NH$_4^+$-N. Interestingly, calculous plant such as Potamogeton crispus showed higher tolerance to NH$_4^+$-N concentration than non-calculous plant such as Vallisneria natans (e.g. Cao et al., 2004, 2007). We speculated the possible reason was that plant calcification alleviated the increase of solution pH, corresponding mitigated the toxic effect of high NH$_4^+$-N.

And we argue that the mitigating effect of increased HCO$_3^-$ on NH$_4^+$-N toxicity is dependent of plant calcification, and it is a positive feedback mechanism, potentially leading to the dominance of calcareous plants in meso-trophic waters in field conditions. Further studies on the function of calcification in relation to carbon–nitrogen metabolism must be carried out in details.

5. Conclusion

In an open experimental system, we confirm that calcification of Chara plants is dependent of the utilization of DIC, and calcification to photosynthesis ratio varies in relation to solution pH (between 9 and 10). External HCO$_3^-$ decreases the increase of solution pH by increasing calcification. And we could conclude that calcification of charophyte plant and other vascular plants alleviate the toxicity of NH$_4^+$-N by regulating solution pH.

Acknowledgements

We would like to express our appreciation to Dr Zhong A.W for her assistant in the measurement of Ca$^{2+}$ content. And we were very grateful to two anonymous reviewers for their valuable comments and suggestions greatly improve the quality of our
manuscript. This research was supported by China Postdoctoral Science Foundation (20090451088) and the National Natural Science Foundation of China (31000161).

References


H. Wang et al. / Chemosphere 93 (2013) 373–379